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Animal origin of 13th-century uterine vellum revealed using noninvasive peptide fingerprinting

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Tissue-thin parchment made it possible to produce the first pocket Bibles: Thousands were made in the 13th century. The source of this parchment, often called "uterine vellum," has been a long-standing controversy in codicology. Use of the Latin term *abortivum* in many sources has led some scholars to suggest that the skin of fetal calves or sheep was used. Others have argued that it would not be possible to sustain herds if so many pocket Bibles were produced from fetal skins, arguing instead for unexpected alternatives, such as rabbit. Here, we report a simple and objective technique using standard conservation treatments to identify the animal origin of parchment. The noninvasive method is a variant on zooarchaeology by mass spectrometry (ZooMS) peptide mass fingerprinting but extracts protein from the parchment surface by using an electrostatic charge generated by gentle rubbing of a PVC eraser on the membrane surface. Using this method, we analyzed 72 pocket Bibles originating in France, England, and Italy and 293 additional parchment samples that bracket this period. We found no evidence for the use of unexpected animals; however, we did identify the use of more than one mammal species in a single manuscript, consistent with the local availability of hides. These results suggest that ultrafine vellum does not necessarily derive from the use of abortive or newborn animals with ultrathin hides, but could equally well reflect a production process that allowed the skins of maturing animals of several species to be rendered into vellum of equal quality and fineness.

pocket Bible | parchment | vellum | collagen | mass spectrometry

> Hamlet: Is not parchment made of sheepskins?
>
> Horatio: Ay, my lord, and of calfskins too.
>
> *Hamlet, V.i*

One of the outstanding controversies in the field of codicology concerns the origin and production of so-called "uterine vellum." Researchers in the field of manuscript studies have long disputed the origin of this ultrafine writing material. Some older scholarship suggested that uterine vellum probably derived from the hides of smaller, more thin-skinned mammals, such as rabbits or squirrels (1, 2). However, most paleographers continue to view the notion of medieval uterine vellum as a myth, on the grounds that its production on the scale implied by extant manuscripts would have entailed an untenably high number of aborted fetuses (3). Other proposed solutions to the derivation of this material have involved specific production processes, such as the splitting of skins (4). The importance of analyzing parchment carefully to determine its origin species and, more specifically, the origin of uterine vellum is a priority research question for several disciplines. Such analysis might not only settle the long-standing debate over the source of supposedly "aborted" hides but would also provide valuable data about the localization and distribution of hides, and thus of membrane books and documents (5). The scholarly disputes notwithstanding, uterine vellum must represent either (*i*) the selection of specific animals whose skin was uniquely fine or (*ii*) the development of craft

Significance

This study reports the first use, to our knowledge, of triboelectric extraction of protein from parchment. The method is noninvasive and requires no specialist equipment or storage. Samples can be collected without the need to transport the artifacts; instead, researchers can sample when and where possible and analyze when required. The level of access we have achieved highlights the importance of this technique. For this study, we have extracted proteins from 513 parchment samples, used to resolve the long-standing question of the origin of "uterine vellum." We find no evidence of unexpected species, such as rabbit or squirrel. We suggest that uterine vellum was often an achievement of technological production using available resources, and would not have demanded unsustainable agricultural practices.

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skills to work a wide range of skins into ultrafine sheets, or, of course, both at once.

The most frequently cited examples of uterine vellum are the 13th-century Paris Bibles (1, 2, 4, 6, 7). These books were single-volume Bibles (pandects) with a consistent organization, which meant that they were easily searchable, making them the ideal reference guide for study and sermon preparation (8). One of the most important subgroups of 13th-century Paris Bibles is the pocket or portable Bible, volumes sufficiently small to be easily transported. Ruzzier (6) suggests that the total output of portable Bibles in the 13th century could have exceeded 20,000 copies. The majority of surviving copies (~54%) were made in France, most in Paris (6), although Bibles of this style were also produced in England at the same time and slightly later in both Italy (notably in the Veneto) and Spain.

To explore the question of uterine vellum, we examined the animal composition of ultrafine parchment (which ranged in thickness from 0.03–0.28 mm in our selected samples), sampled primarily from pocket Bibles, and compared these samples with other parchments that book-end their main period of production. If the skins of small animals (rabbits and squirrels) were used, their presence would be revealed by zooarchaeology by mass spectrometry (ZooMS). If the production of uterine vellum represented instead a specialized craft skill, then the selection of animals would be similar to the selection of animals evident in other coarser membranes from the same geographic region.

Species Identification of Parchment

To assess the origin of uterine vellum, the principal line of evidence is the identification of the skin. Skins have previously been identified by overall size, thickness, color, levels of grease, and patterns of follicles (9). A notable proponent of follicle pattern analysis was Ryder (10), who used the technique to identify animal species; however, not all parchments had discernible follicle patterns, not every pattern could be identified, and paleographers have often been overconfident in their ability to discern species origin from such patterns. Protein (11, 12) and DNA-based methods (13–17) potentially offer absolute determination of species but, until now, have had other limitations. Toniolo et al. (11) analyzed 5 mg of parchment from the 13th-century "Marco Polo Bible" at the Biblioteca Medicea Laurenziana, and used peptide sequences to identify a single leaf as calfskin, although if more leaves had been analyzed the results may have revealed different species as was the case with the family's archival documents (Table S1). Kirby et al. (12) also used destructive sampling techniques to analyze a Koran and other objects. Teasdale et al. (13) have recently reported genomic data from postmedieval parchment using, on average, 50 mg of parchment. In addition to the destructive nature of sampling, a further hidden cost of molecular analysis is adequate storage of extracted samples. It is usually necessary to freeze DNA and protein extracts to preserve sample integrity, which adds a further cost burden for libraries and archives in either shipping or storage.

Triboelectric Extraction

Here, we describe a novel noninvasive molecular identification method to identify the origin of parchment, using electrostatic molecular extraction onto a solid-phase PVC polymer. PVC polymer erasers are widely accepted by the conservation community as a noninvasive measure for removing dirt. Indeed, every archive and conservation studio has access to and experience of the use of PVC erasers. A further advantage of the extraction method is that protein is preserved on the PVC polymer waste at room temperature with no further storage requirements (apparently indefinitely). It can therefore be retained at the point of collection without special preparation or storage until the researcher deems it appropriate to analyze a larger set of samples. The method is easily scalable and places the sampling in the hands of curators, codicologists, and conservators. Using this approach, we have sent kits (consisting of PVC erasers, nitrile gloves, and sampling tubes) to 14 archives and 40 libraries in Europe and North America. They have returned samples from 79 13th-century pandect Bibles for analysis, including 72 pocket Bibles.

The aim of this study was to identify the animal origin of the tissue paper-thin "uterine" vellum, used in 13th-century pocket Bibles, through the use of a novel noninvasive triboelectric extraction of skin collagen, subsequently analyzed by conventional peptide mass fingerprinting.

Results

Validation of Triboelectric Extraction. To assess the quality of our results, we performed a comparative analysis of the same sample using conventional ZooMS techniques that require destructive sampling (12), as well as nondestructive electrostatic ZooMS (eZooMS). For the purpose of this experiment, we used two different documents: (*i*) a 16th-century manorial court roll (Fig. 1*A*) and (*ii*) an 18th-century seal tag (Fig. 1*B*). In case *i*, a fragment of parchment of ~0.5 cm × 0.5 cm was used, and in case *ii*, a fragment of ~0.1 cm × 0.3 cm was used. In both cases, an eZooMS sample was taken from the main document.

The optimization of our eZooMS methodology has allowed us to obtain equal, if not better, results from PVC sampling than when using an actual fragment of parchment (Fig. 1). The observed difference in quality is probably due to an excessive amount of collagen being extracted during destructive analyses (Fig. 2). However, the eraser technique itself may also contribute

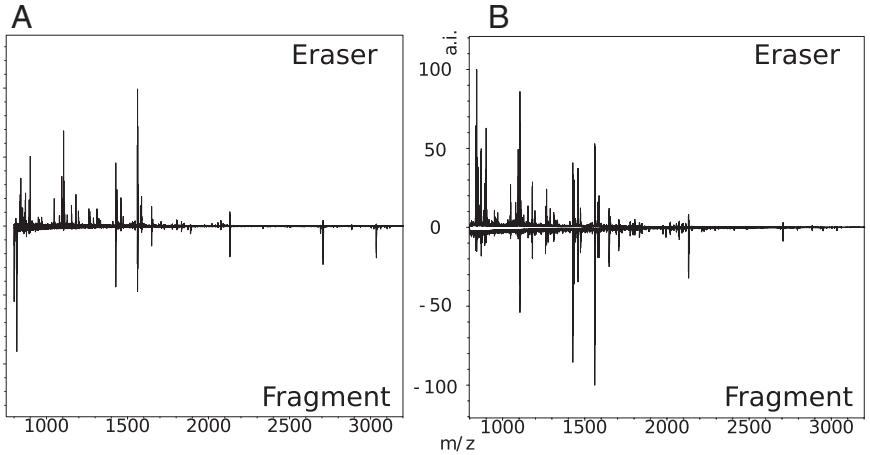

Fig. 1. Comparison of peptide mass fingerprint from samples *A* and *B* using destructive sampling (lower spectra) and noninvasive eZooMS sampling (top spectra). (*A*) For one sample, the resulting peptide mass fingerprint detected fewer peaks than in its equivalent eZooMS sample. (*B*) In the other sample, the results obtained were very similar for both methods. eZooMS samples extracted and analyzed 12 mo apart, having been stored at room temperature, revealed no loss of signal.

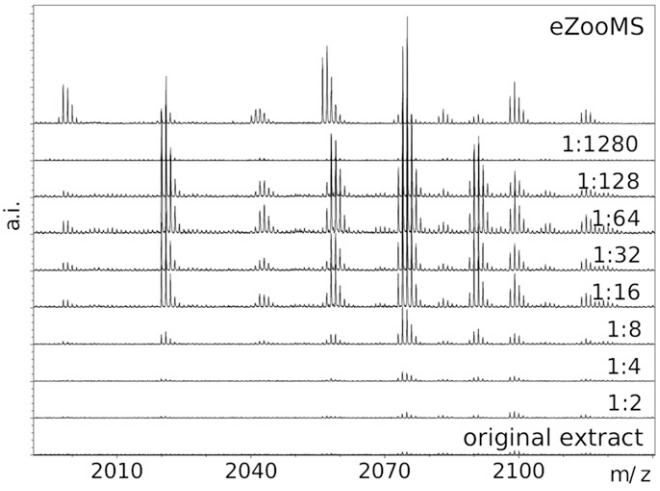

Fig. 2. Comparison of peptide mass fingerprint from serial dilution of destructive sample and noninvasive eZooMS sampling. A destructive sample from historic calf parchment (0.5 cm × 0.5 cm) was extracted and digested using a serial dilution ranging from 1:2–1:1,280 and compared with a noninvasive eZooMS extraction. A cleaner signal is seen between the 1:16 and 1:128 dilutions, likely due to the dilution of excessive collagen molecules or contaminants that interfere with the subsequent analysis.

to a cleaner signal by the eraser retaining contaminating molecules that interfere with the subsequent analysis on the PVC.

Species Variation. Species identifications of all of the samples analyzed are included in Tables S2 and S3. A total of 220 folios were analyzed from the 72 pocket Bibles. Of these folios, 68% were calf, 26% were goat, and 6% were sheep. We found no evidence for the rabbit skin duodecimos suggested by Pollard (1). Of the 72 Bibles we sampled, 62 were sampled on multiple leaves. In most cases, the identification was consistent for each of the folios of one manuscript, including the 18 sampled leaves of the Hornby Bible (OSU.MS.MR.Frag.74). However, at least five Bibles were composed of parchment from multiple species. To confirm the presence of multiple animals, we were able to analyze 20 folios from Cambridge University Library Ee.6.26, six of which were identified as calf and 14 as sheep. The species distinction is mirrored by stylistic differences within the Bible, which suggest that this manuscript may, in fact, be a composite of two different Bibles. The first part of the Bible (fol. 1–108), which contained five folios identified as calf parchment, resembles a "proto-'Paris' Bible" model produced *ca.* 1200–1230, whereas the second part (fol. 109–459), containing the 14 folios made of sheepskin (and one of calfskin) parchment, is a far better fit for the "mature 'Paris' Bible" blueprint of *ca.* 1230–1280 as described by Light (8, 18–20). It is also worth noting that the attributed English provenance derives from inscriptions present in the second part of the Bible; no provenance indicators are recorded for the first part, so it is possible that the Bible's first 108 folios were produced in France.

In addition, we explored the possibility of using liquid chromatography-tandem MS to determine if differential biomarkers for uterine skin could be identified (*SI Materials and Methods*). Modern uterine samples showed elevated expression of four proteins (Fig. S1); however, none of the peptide markers observed in eZooMS mapped to any of these proteins, so it is not possible to report the presence of these proteins in our Bible samples.

Geographic Distribution. Previous authors have identified geographic variation in the animals used for making parchment. Forbes (21), citing Wattenbach, notes that hides used for parchment were predominantly calf in the north of Europe and sheep and goat in the south. Ruzzier (6) agrees, and suggests that fine parchment north of the Alps was likely made from calfskins. Clarkson (4), noting the "warm, creamy tint" and "flexibility" of late-medieval Italian skins, attributes these qualities to the use of goatskin and possibly alternative methods of preparation. De Hamel (3) observes that Italian parchment is often prepared from goatskin, which contrasts with the poor representation of goatskin in England (22–24).

Our results are in agreement with most of these assessments. We see a predominance of calfskin being used in France, a pronounced use of goatskin in Italy, and a more mixed pattern emerging from England (Fig. 3).

To examine whether the geographic variation observed for ultrafine parchment differed from coarser membranes, we surveyed an additional 293 parchment objects from the 12th to 14th centuries. The selection of skins appears to reflect available livestock, and therefore a city's or a region's preferences: Sheepskin is most abundant in England, calfskin in France, and goatskin in Italy. In Italy, there is an absence of goatskin in the 12th century (contra 25), which possibly reflects regional variation. Six (of 12) 12th-century Italian sheep samples were sourced from Sicily, where a sheep-based dairy economy was practiced. Although there are differences between the two centuries spanning the period of interest, a more comprehensive investigation is required to study any long-term changes.

The pattern of selection of ultrafine parchment for Bible production seems to be broadly in line with the pattern of selection found in other parchment analyzed (Fig. 3). The English Bibles seem to be more varied in their composition, including multiple species in the same volume. Of the five Bibles that were composed of a mixture of animals (calf and goat or calf and sheep), three of

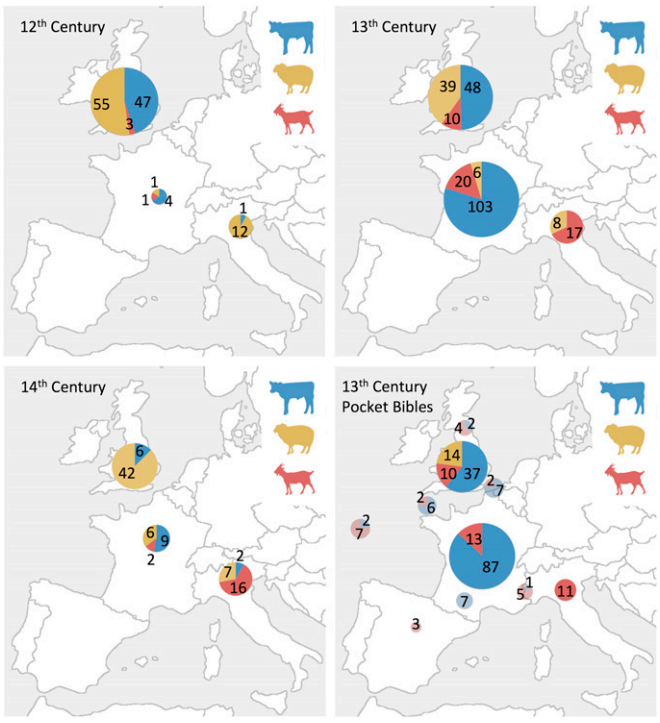

Fig. 3. Relative proportions of animals used to make parchment in each of the three regions studied (France, England, and Italy) during the 12th to 14th centuries. The size of the circle indicates the number of samples. Except for the figure describing exclusively pocket Bibles, data were obtained from all sources of parchment, including legal documents, secular codices, and Bibles, using the eZooMS method. Circles shown in paler colors indicate inconclusive provenance with respect to location.

them are of English provenance, one of possibly English provenance, and the fifth of either French or Italian provenance. Of the seven larger Paris Bibles (i.e., classified as nonpocket pandect Bibles), the three described as being of French production, as well as the two Bibles from the Low Countries and Germany, were all identified as calf; however, the two larger Paris Bibles of English origin were found to be made from sheep. All these nonpocket Bibles also seem to follow general regional preferences.

The scant use of sheepskin parchment for pocket Bibles is provocative and merits further investigation. A survey of English archival documents from the Borthwick Archive (York), using the same methods described in this paper (Table S4), reveal that all are written on sheepskin parchment. This selection of sheepskin parchment for legal documents may not merely reflect cost. The *Dialogus de Scaccario* (26) argues for the use of sheepskins as the medium for English legal documents due to the difficulty of erasure of text as a result of the propensity of sheepskin parchment to delaminate. This characteristic also means that sheepskin is the easiest skin to split, apparently making it ideally suitable for the thin parchment used in pocket Bibles. However, apart from the two larger Paris Bibles of English origin, we have found only one example of a pocket Bible made of sheepskin: This pocket Bible is of supposed English provenance (Cambridge University Library Ee.6.26).

Our results have shown that pocket Bibles, some of the first examples of commercial book production (8), were written on all three species used widely in parchment production. In our survey of 220 folios spanning three centuries, no unexpected species were recorded. The use of sheepskin in only one of 72 pocket Bibles indicates that it was not favored for these very thin membranes despite the facts that sheepskin delaminates and that the range of thicknesses measured for calf and sheep parchment are similar (0.09–0.28 mm for calf and 0.07–0.26 mm for sheep) (Fig. 4*D*). The presence of goatskin and sheepskin parchment in the sample set would seem to indicate that uterine calfskin was not necessarily used to produce very fine membranes. Indeed, similar thickness measurements can be achieved for all three species (Fig. 4*A*).

Discussion

Aside from local availability of livestock and preferences in meat consumption, parchment production in medieval Europe was limited by logistical challenges. Parchment production was presumably located close to the point of slaughter, because skins deteriorate within days, resulting in poor quality and spotty membranes (2). Hides can be preserved by salting, but this process would be prohibitively expensive in Northern Europe: The salt price in England rose from a base above 0.1 g·Ag·kg^{-1} during this period.

In addition, the prestige and value of particular types of skin played an important role. Fourteenth-century accounts from Beaulieu Abbey (27) show that calfskin was more highly valued than sheepskin. The importance of the hide as a source of revenue is repeatedly evidenced by the abundance of skinning marks on animal remains, the range of decrees and bylaws restricting the flaying of animals that had died of diseases, and the lengths the authorities went to prevent these skins being used (28). The intact skeleton of a cow from a 14th-century burial at Téteghem Carlines 3 (Northern France) that died of a dystocic calving (29) gives a further insight into the importance (or otherwise) of uterine hides. Although the cow's hide had been removed, the calf remained trapped and unflayed in the birth canal, despite the fact that it could have been easily released and flayed to obtain uterine calfskin for parchment.

However, most of the geographic variations can be explained by the local livestock availability and preferences in meat consumption in the different regions.

France. The relatively low use of goat parchment in Parisian pocket Bibles may be explained, in part, by the patterns of consumption. In *Le Viandier de Taillevent* (a recipe book first produced in the early 14th century), of 140 recipes, veal appears in seven (5%). Kid and lamb are listed in only one recipe and are considered interchangeable depending on availability. Evidence from the 15th century suggests that sheep and goats were much more common in Southern France (30). Excavations in multiple sites from the Parisian area revealed 3,102 bone fragments dating to the 13th century, the majority of which were caprines (sheep/goat, 38.2%) followed by similar levels of ∼30% cattle and pig (31).

Although the species of animal used would reflect local livestock availability, the age of the animals was limited by the craft production. La Lande's book *Art de faire le parchemin*, written in 1762, indicates that calfskins suitable for making parchment should not be taken from an animal older than 6 wk of age (32). The skins of young calves, although suitable for parchment production, are already several times thicker than the skin of adult goats or sheep. Although adult goats and sheep can be used for the production of certain types of parchment, adult cow skins are too thick for parchment production and are instead used to produce heavy, durable leather (33). However, Clavel (31) notes that calf bones are rarely found in 13th and 14th century sample collections and comprise only 2–5% of all cattle bones from excavated sites, appearing with greater frequency in later periods. Veal

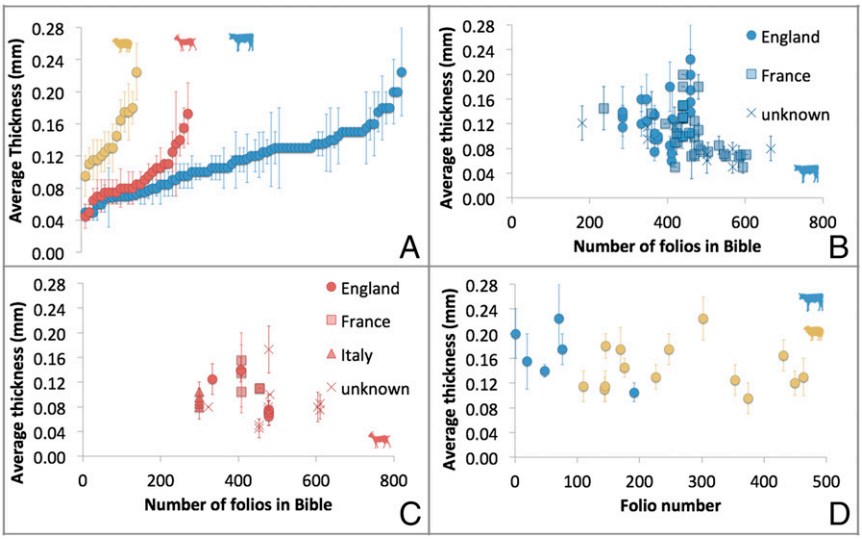

Fig. 4. Thickness of folios. (*A*) Thickness of all pocket Bible folios by species. (*B*) Thickness of calf pocket Bible folios by total number of folios in Bible. (*C*) Thickness of goat pocket Bible folios by total number of folios in Bible. (*D*) Thickness of folios from pocket Bible Ee.6.26.

calves were typically slaughtered at around 6 mo of age, but the bones of very young cattle have rarely been found in French medieval archaeological sites (34).

England. The presence of goatskin in the English pocket Bibles is intriguing, an assertion that is further supported for one sample with DNA evidence (Fig. S2). Goatskin folios produced in England are thinner than their Italian or French counterparts (Fig. 4*C*). This evidence would seem to indicate that goatskin was not routinely available and may have been acquired specifically for the production of pocket Bibles. However, goat horn-core accumulations are not unusual, particularly in the eastern part of the country and in urban sites, possibly the product of an international skin trade, because horns were routinely left within the transported skins (22). In both early and later medieval England, sheep are overwhelmingly more common than goats; when identifiable, the latter invariably represent less than 10% of the total sheep/goat bone and tooth fragments, and this proportion is often close to 1%. Goat bones occur on ∼50% of sites, but invariably in small frequency. In the late-medieval period, both documentary (35) and archaeological (34) sources attest to a further decline in the use of goat.

Cattle were predominantly slaughtered as adults in medieval England, which reflects their major role as tractors. The gradual replacement of working cattle with horses toward the end of the period, at least in some regions, provided the opportunity for an increase in early culling. From the 15th century onward, several sites, mainly urban, have produced relatively high proportions of calf bones, probably a consequence of an increased demand for veal and milk, as well as a greater production of calfskins (34, 36), but this evidence does not explain the predominant use of calfskin in English pocket Bibles in the 13th century.

Italy. At Italian archaeological sites, sheep bones also tend to predominate during the medieval period, but far less so than in England, with a typical sheep/goat ratio ranging between 3:1 and 2:1 (37–39). Italian zooarchaeological reports, however, rarely provide specific identifications for caprines, perhaps due to the difficulty in separating sheep from goat morphologically. The predominance of goat horn-cores is not a phenomenon known for Italian sites.

The use of cattle is clearly variable, with emphasis on meat production or traction, depending on the site. The presence of very young animals is, however, reported at several urban (39) and high-status sites (40). However, the nature of the current evidence is insufficient to allow us to identify any clear chronological trends.

Conclusion

This study reveals the value of the triboelectric eZooMS approach to the analysis of parchment. It requires no specialist equipment or storage, samples can be collected when appropriate without the need to transport the artifacts, and the samples can then be analyzed when required. As we have shown (Fig. 1), triboelectric extraction is more efficient than physical sampling for four reasons. First, much less material is required. Second, the molecular extraction is bulked onto a large volume of eraser waste, which means it is easy to subsample. Third, molecules are stabilized on the surface of the eraser waste. Fourth, eZooMS acts as a prepurification step, as evidenced by the results from whole samples. Following extraction, the pigmented extracts remain on the eraser crumbs and only the macromolecules are extracted.

We have been able to provide the first significant molecular evidence, to our knowledge, to resolve the long-standing question of the origin of uterine vellum. We find no evidence of unexpected species, such as rabbit or squirrel. Although this type of parchment derived mostly from calfskin in France, in other places, different skins were used subject to local availability. The production of ultrathin parchment was the result of a technological production process using available resources and, as such, would not have demanded unsustainable agricultural practices. We have further shown that manuscript documents made from animal skins are a valuable additional resource for archaeologists exploring livestock economies.

Although the use of genuine uterine vellum cannot be discounted, our results suggest that its availability was not a defining factor in medieval parchment production. Instead, our findings would seem to emphasize dependence on a highly specialized craft technique rather than the supply of a particular raw material. A more likely explanation for the production of fine parchment is the use of relatively young animals and the deployment of specific finishing techniques that enabled the corium to be ground to the desired thickness. The density of collagen fibrils in calf and goat parchment, compared with a more open weave and higher fat content in sheep parchment, favors the former two species; nevertheless, it is evident that parchment makers had the skills to make the finest parchments from all three.

Materials and Methods

All codices sampled were classified in their catalog entries as 13th-century Bibles. The 79 Bibles were subdivided, depending on their dimensions, into two groups based on criteria used by Ruzzier (6): pocket Bibles (height + width < 385 cm) and nonpocket Bibles (height + width >385 cm). Details of all Bibles sampled can be found in Tables S2 and S3.

Sampling. Samples were extracted in the participating archives and libraries using kits sent by one of the authors (S.F.) consisting of 1.5-mL microcentrifuge tubes, nitrile gloves, acid-free paper, and nonabrasive conservator's erasers. Sampling was performed using a Staedtler "Mars Plastic" eraser, rubbing the eraser in one direction and collecting the resulting eraser waste fragments in individual 1.5-mL microcentrifuge tubes. For each sample, a new individual piece of eraser and acid-free paper was used; the eraser and paper were thrown away once sampling of the folio was completed to avoid cross-contamination. Nitrile gloves were worn throughout the sampling process to avoid keratin (human skin) contamination. Sample collection was undertaken on areas of the document that had no writing and presented structural integrity (absence of holes or tears in the parchment). All samples were stored at room temperature until required, usually by the partner. Details of each document sampled were entered onto an online spreadsheet shared between the partner and the laboratories in York.

eZooMS. Initially, eraser crumb samples [sometimes known as "erdu" (41)] were spun down at maximum speed on a benchtop centrifuge for 1 min and 75 μL of 0.05 M NH_3CO_3 (AmBic) buffer (pH 8) was added to each sample. Samples were heated at 65 °C for 1 h. Once cooled, 1 μL of trypsin (0.4 μg/μL) was added and samples were incubated at 37 °C for 18 h. However, at a later stage, the method of collagen extraction was optimized by removing the heating step and condensing the process into one incubation step at 37 °C for 4 h with both AmBic and trypsin added simultaneously. After incubation with trypsin, digests were spun down at maximum speed on a benchtop centrifuge for 1 min and 1 μL of 5% (vol/vol) TFA was added. Samples were desalted and concentrated using C18 resin (Millipore), following the manufacturer's instructions. Peptides were eluted in a final volume of 50 μL of 50% acetonitrile (ACN)/0.1% TFA (vol/vol). One microliter of eluted peptides was mixed on a ground steel plate with 1 μL of α-cyano-4-hydroxycinnamic acid matrix solution [1% in 50% ACN/0.1% TFA (vol/vol/vol)] and air-dried. All samples were spotted in triplicate. Samples were analyzed using a calibrated Ultraflex III (NLD1; Bruker Daltonics) MALDI-TOF instrument in reflector mode. Spectral analysis was performed using the open-source cross-platform software mMass (www.mmass.org) (42), and individual peptides were identified manually according to Buckley et al. (43, 44).

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